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13. ABSTRACT (Maximum 200 Words)

During past year, I have focused my on AKT2 regulation of MST1 pathway and the effort of AKT2-interaction protein, APBP/APαB, on AKT2 signaling. MST1 is a member of STE20 like kinase and has been shown to induce apoptosis through its cleavage, activation and nuclear translocation. I have shown that AKT2 interacts with and phosphorylates MST1 at threonine-120, resulting to inhibition of MST1 cleavage and nuclear translocation. Thus, AKT2 abrogates MST1-induced apoptosis. AKT2-interaction protein, APBP/APαB, mediates AKT2 activation of p21-activated kinase 1(PAK1) pathway to induce cell survival.

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Introduction

The purpose of this project is to: 1) Examine the incidence and clinical significance of AKT2 alterations in breast cancer, 2) Define the functional interaction between AKT2 and APBP/APαB and role of AKT2/APBP in mammary epithelial cell transformation and 3) Determine the FTIs as an inhibitor of AKT2 pathway for breast cancer intervention.

Body:

During the last budget year, we have mainly focused on AKT2 regulation of MST1 kinase and the effects of APBP/APαB on AKT2 signaling

1. Akt Phosphorylates Mammalian Hippo Ortholog MST1 to Attenuate Apoptosis through Inhibition of its Cleavage and Kinase Activity

The *MST1*, a mammalian homologue of the Drosophoila *Hippo* (1-3), encodes serine/threonine kinase that is cleaved, activated and translocated to the nucleus upon apoptotic stimulation (4). MST1 induction of apoptosis was thought to be mediated by activation of JNK/p38 and/or decrease IAP family proteins (1-4). Regulation of MST1, however, is currently unknown. Here we show that the cleavage of MST1 induced by DNA damage or FasL is inhibited by IGF-1 through phosphatidylinosotol 3-kinase (PI3K)/Akt pathway. Ectopic expression of constitutively active PI3K catalytic subunit p110α or Akt attenuated MST1 cleavage and MST1-mediated apoptosis (Fig. 1). Akt interacts with (Fig. 3) and phosphorylates MST1 at threonine 120 (Fig. 2), which leads to inhibition of its cleavage, kinase activity and nuclear translocation (Figs. 4 and 5). Instead, the phosphorylated MST1 binds to 14-3-3 protein (Fig. 4). Further, we demonstrate that MST1-activated JNK feedback phosphorylates MST1 at serine 82, enhances MTS1 activation (Fig. 6) and promotes MST1 nuclear translocation (Fig. 7). The phopshorylation of MST1 by Akt inhibits both MST1-induced activation of JNK and constitutively activated JNK phosphorylation of MST1 and induced MST1 nuclear translocation (Fig. 6 and 7), suggesting that Akt is a key regulator of MST1/Hippo signaling.

2. APBP/APαB, an AKT2-interaction protein, promotes cancer cell growth and survival by mediating AKT2 activation of p21-activated kinase 1 (PAK1).

To further understanding molecular insight of AKT2 in human carcinogenesis, we identified an AKT2 interaction protein, APBP/APαB by screening yeast two-hybrid library. APBP/APαB contains four AKT2 phosphorylation consensus sites, a SoHo motif and three SH3 domains (Fig. 8). APBP/APαB binds to C-terminus of AKT2 through its first and second SH3 domains (Fig. 8). It also interacts with PAK1 (Fig. 9), indicating the SH3 domains of APBP/APαB as docking sites for AKT2 and PAK1. AKT2 phosphorylates APBP/APαB *in vitro* and *in vivo* (Fig. 10). Expression of APBP/APαB induces PAK1 activity and overrides apoptosis induced by ectopic expression of Bad or DNA damage (Figs. 9 and 11). Nonphosphorylatable APBP/APαB-4A and SH3 domain truncated mutant APBP/APαB attenuate constitutively active Akt-induced PAK1 activation and inhibit AKT2 and PAK1 phosphorylation of Bad and antiapoptotic function (Fig 12). These data indicate that APBP/APαB is a physical substrate of AKT2, functions as adaptor for AKT2 and PAK1, and plays a pivotal role in AKT2/PAK1 and NFκB cell survival and growth pathways.

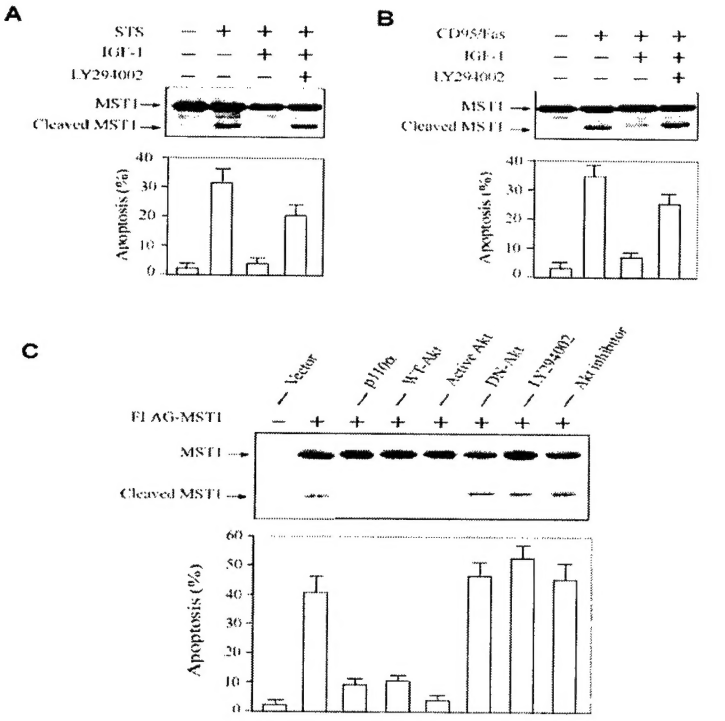

Fig. 1. Apoptotic stimuli-induced MST1 cleavage and programmed cell death are inhibited by activation of PI3K/Akt pathway. (**A** and **B**) COS7 cells were treated with indicated reagents and immunoblotted with anti-MST1 antibody (top panel). Apoptotic cells were determined by Tunel assay and quantified (bottom). (**C**) COS7 cells were transfected with Flag-MST1 (+) and Akt or p110*a* expression plasmid. After 24 h transfection, cells were treated with VP16 together with PI3K inhibitor LY294002 or Akt inhibitor for 10 h and subjected to immunoblotting (top) and Tunel assay (bottom) analyses.

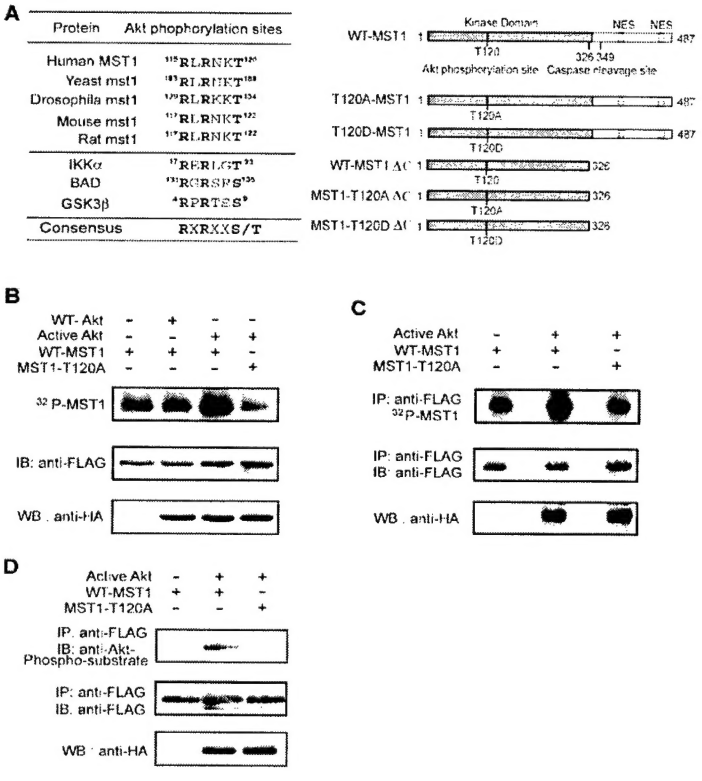

Fig. 2. Akt phosphorylates MST1 at residue threonine-120. (**A**) Comparison of the putative Akt phosphorylation sites in MST1 with the sequences of phosphorylation sites of known Akt substrates. (**B**)*In vivo* labeling (top). Akt phosphorylates MTS1 *in vivo*. Expression of Flag-MST1 was shown in bottom panel. (**C**) *In vitro* Akt kinase assay revealed Akt phosphorylation of threonine-120 of MST1 (top). Expression of MST1 and Akt was shown in middle and bottom panels. (**D**) Immunoblotting analysis of the Flag-MST1 immunoprecipitates with anti-Akt-phopsho-substrate antibody (top). Middle and bottom panel shows expression of Flag-MST1 and HA-tagged constitutively active Akt.

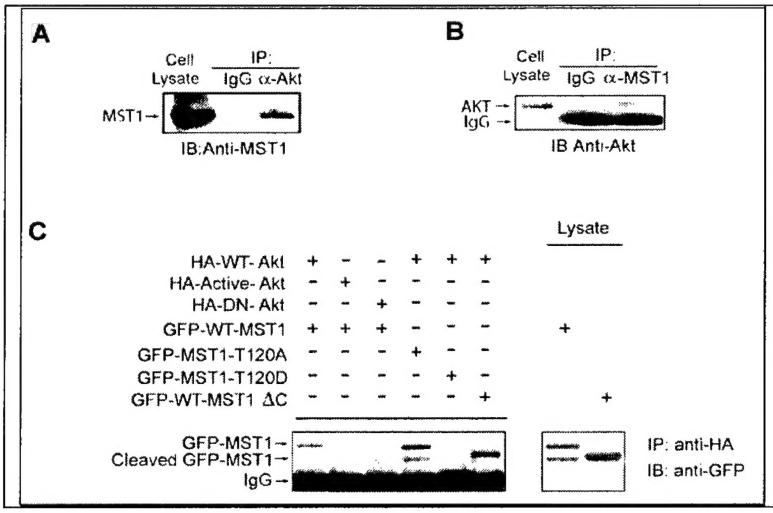

Fig. 3. Akt interaction with MST1. (**A** and **B**) Immunoprecipitation was carried out with anti-Akt antibody and detected with MST1 antibody (A) and vice versa (B) in HEK293 cells. (**C**) Full-length and cleaved MST1 only bind to wild type Akt. HEK293 cells were transfected with indicated plasmids, immunoprecipitated with anti-HA and detected with anti-GFP antibody.

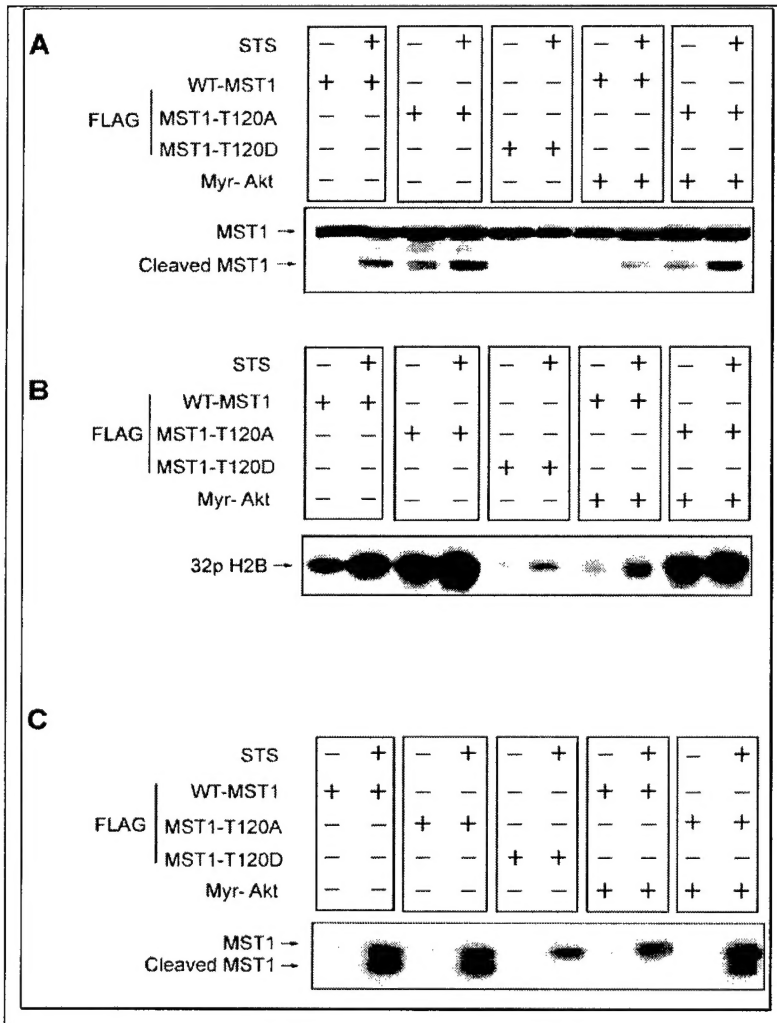

Fig. 4. Akt inhibits MST1 cleavage and kinase activity in phosphorylation of threonine-120 dependent manner. (**A**) COS7 cells were transfected and treated with indicated plasmids and reagent and then immunoblotted with anti-Flag antibody. (**B**) *in vitro* MST1 kinase assay. Following transfection and treatment with indicated plasmids and reagent, COS7 cells were lysed and immunoprecipitated with anti-Flag antibody. The immunoprecipitates were subjected into in vitro kinase assay using histone H2B as substrate. (**C**) In-gel MST1 kinase assay. COS7 cells transfected and treated as indicated on top. MST1 immunoprecipitates were run in the gel that contains MBP substrate. The gel was incubated with a [^{32}P]ATP containing kinase buffer.

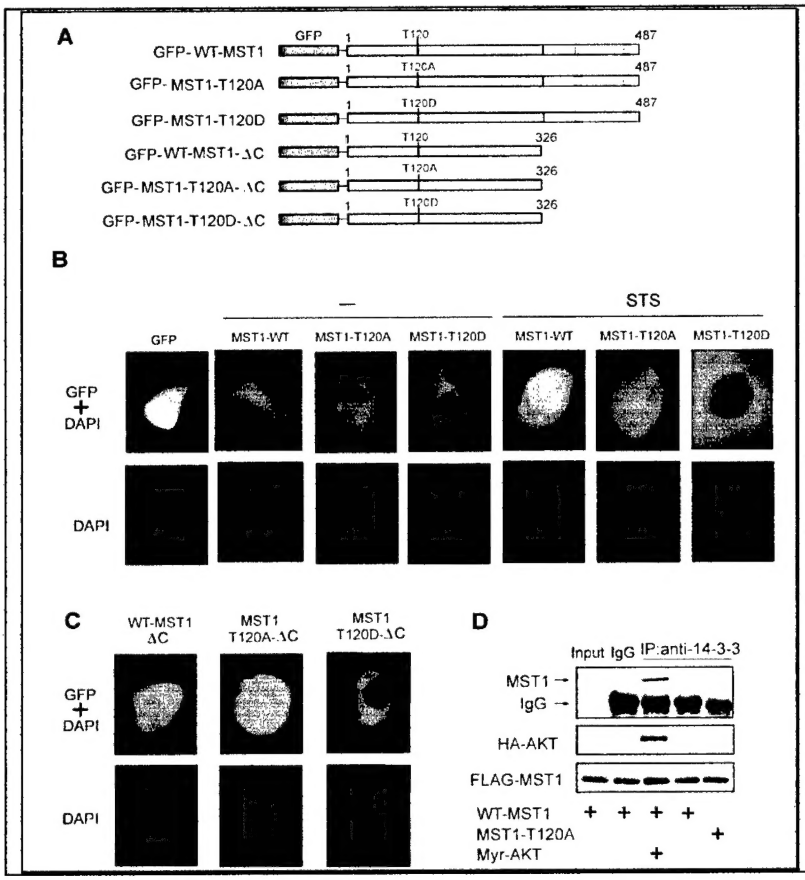

Fig. 5. Akt inhibits MST1 nuclear translocation and promotes MST1 binding to 14-3-3 protein. (**A**) Diagram represents GFP-tagged MST1 constructs. (**B**) and (**C**) COS7 cells were transfected with indicated plasmids and treated with or without STS and visualized under fluorescence microscope. (**D**) HEK293 cells transfected with indicated plasmids, and immunoprecipitated with anti-14-3-3 antibody. The immnoprecipitates were immunoblotted with anti-MST1 antibody (top). Expression of transfected Akt and MST1 was shown in middle and bottom panels.

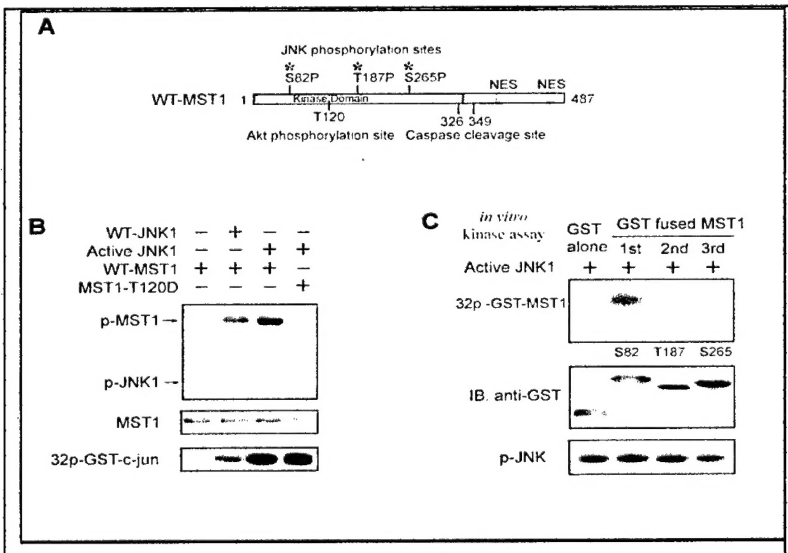

Fig. 6. JNK1 phosphorylates of MST1 at serine-82 that was inhibited by Akt. (**A**) Diagram indicates 3 potential JNK1 phosphorylation sites (S/T-P). (**B**) and (**C**) *In vitro* JNK1 kinase assay using full-length MST1 (B) or GST-fused individual putative JNK1 phosphorylation site (C) of MST1 as substrate (top). Middle panels show expression of MST1 and bottom panels are controls showing JNK1 kinase activity and JNK1

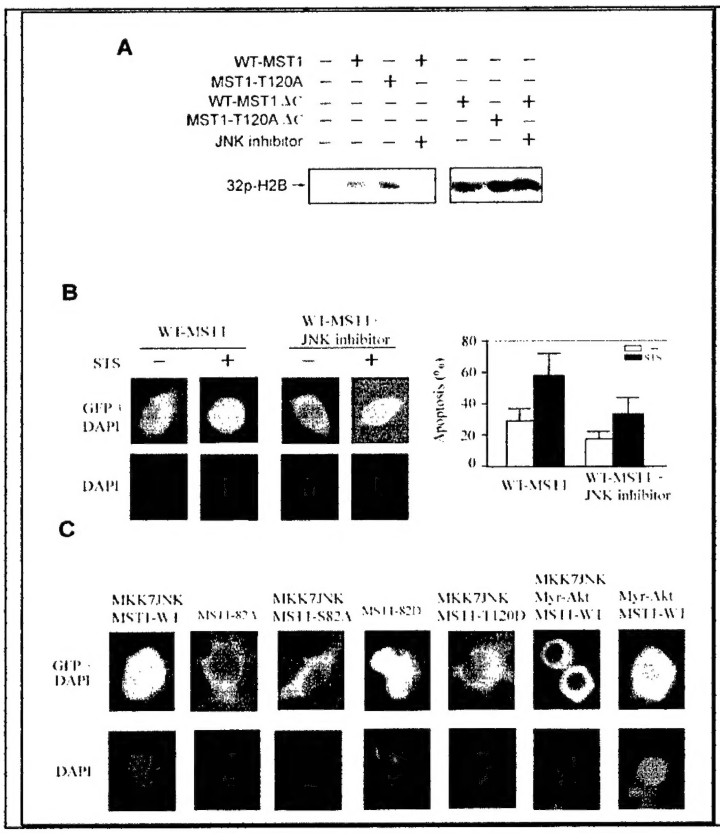

Fig. 7. JNK1 phosphorylation of MST1 threonine-120 induces MST1 nuclear translocation that was inhibited by Akt. (A) In vitro MST1 kinase assay shows that JNK inhibitor partially attenuates full length WT-MST1 activity. (B) and (C) COS7 cells were transfected with indicated plasmids and treated with or without STS and visualized under fluorescence microscope.

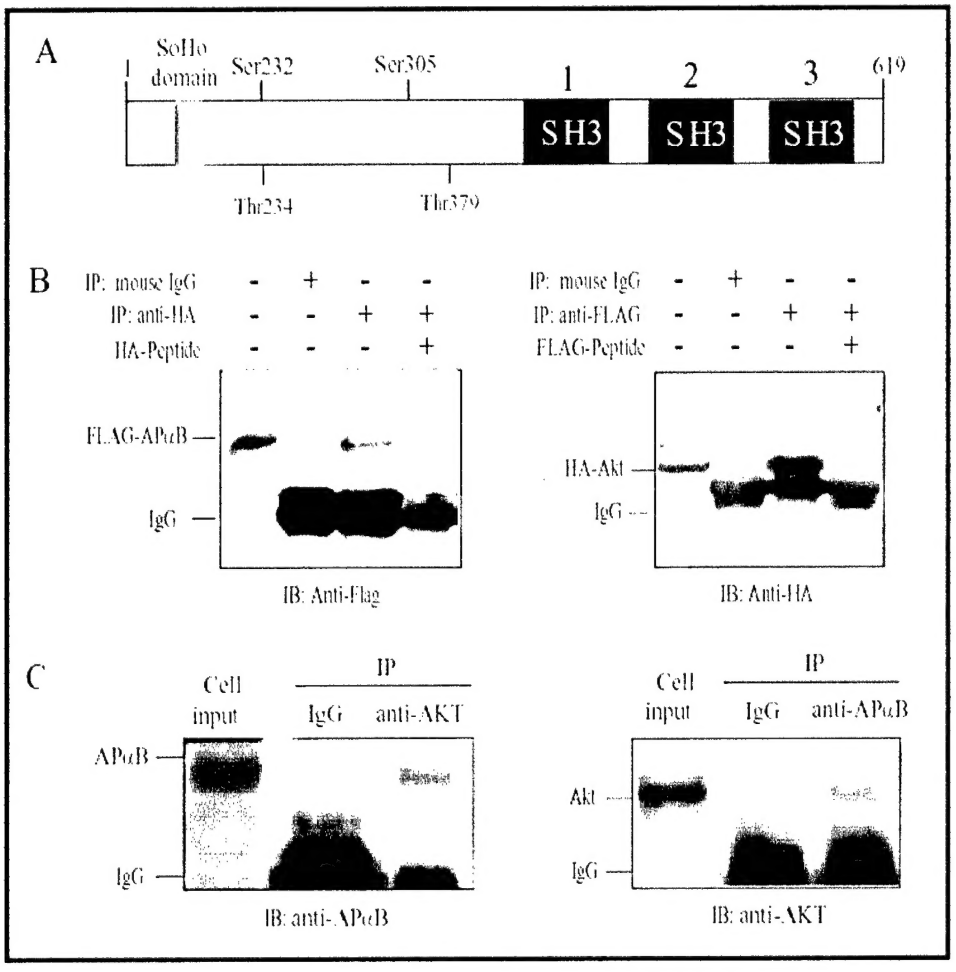

Fig. 8. Akt interacts with APαB. (**A**) Schematic representation of the domain structure and Akt putative phosphorylation sites of APαB. (**B**) Coimmunoprecipitation analysis of HEK293 cells transfected with HA-Akt and Flg-APαB. Immunoprecipitation was performed with anti-HA antibody and detected with anti-Flag antibody (left panel) or vice versa (right panel). (**C**) Association of Akt and APαB at physiological protein concentration. Western blot analysis of the Akt immunoprecipitates prepared from Hela cells with anti-Akt1 antibody and detected with anti-APαB (left panel) or vice versa (right panel).

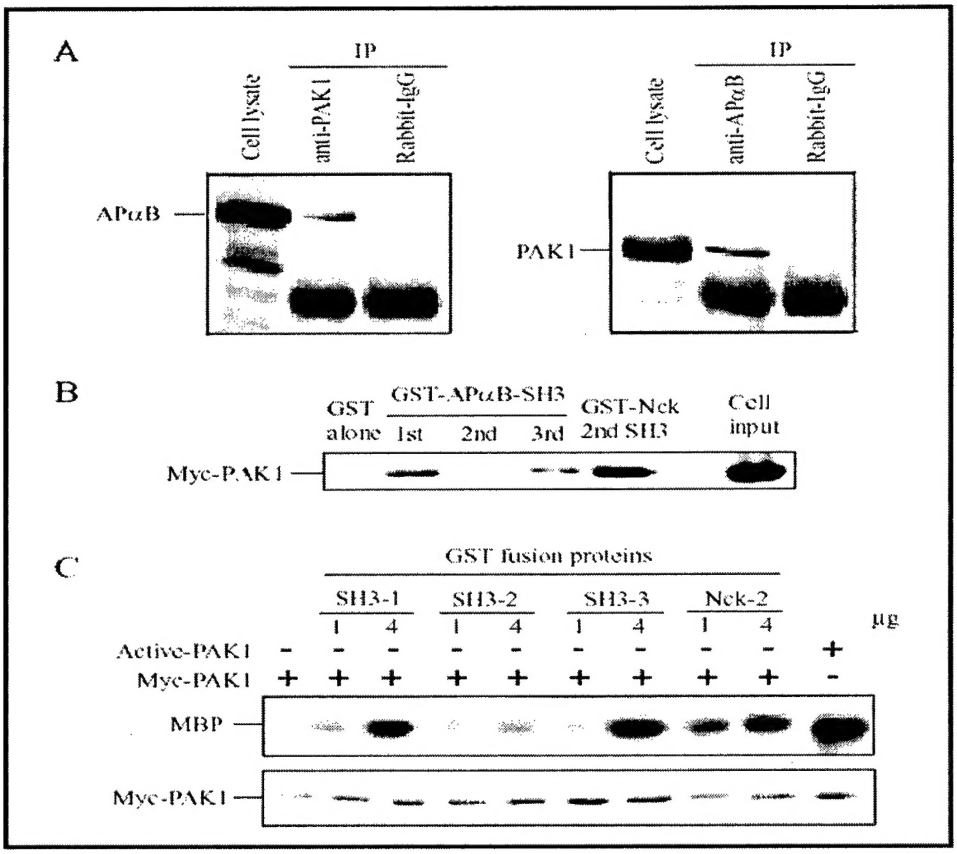

Fig. 9. APαB interacts with PAK1 and directly activates PAK1 *in vitro*. (**A**) Immunoblotting analysis. Hela cells were lysed, immunoprecipitated with anti-PAK1 antibody and detected with anti-APαB antibody (left panel) or vice versa (right panel). (**B**) GST pull down assay. The cell lysates prepared from Myc-PAK1-transfected HEK293 cells incubated with indicated GST fusion proteins. Second GST-SH3 domain of Nck, which is known to binds to PAK1, was used as a positive control. Bound PAK1 was detected by Western blot analysis of GST pull down products with anti-Myc antibody. (**C**) In vitro kinase assay. HEK293 cells were transfected with Myc-PAK1 and immunoprecipitated with anti-Myc antibody. The Myc-PAK1 immunoprecipitates incubated with 1 μg and 4 μg of indicated GST fusion proteins in a *in vitro* kinase buffer containing [^{32}P]ATP and MBP (top panel). Expression of transfected PAK1 was shown in the bottom panel.

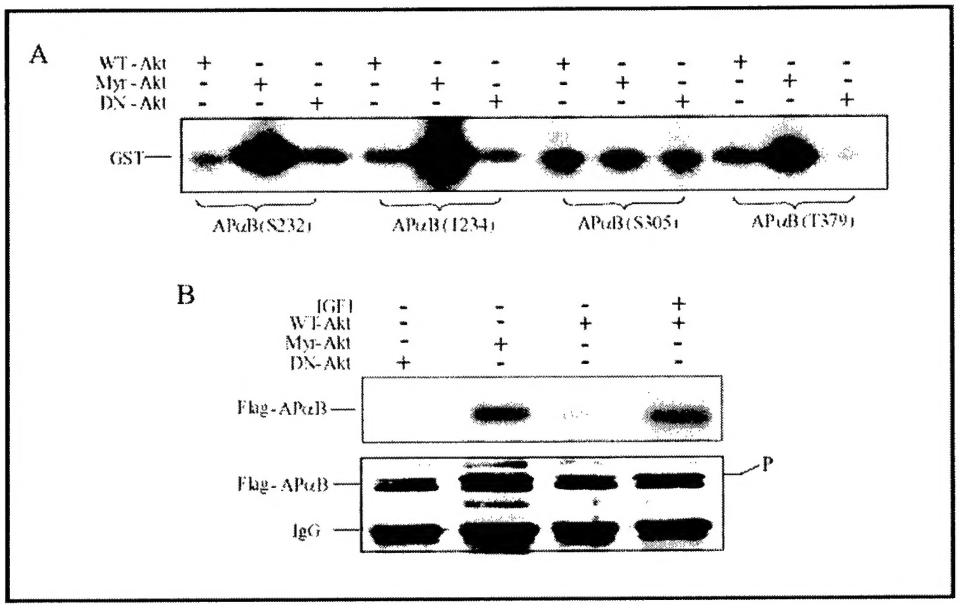

Fig. 10. Akt phosphorylates APαB *in vitro* and *in vivo*. (**A**) *In vitro* kinase assay. HEK293 cells were transfected with indicated Akt expression plasmids and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected into kinase assay using GST-fused each putative Akt phosphorylation site of APαB as substrate as indicated below the figure. (**B**) *In vivo* [^{32}P]orthophosphate cell labeling. COS7 cells were transfected/treated with indicated plasmids and reagent and incubated with [^{32}P]orthophosphate for 4 h. Immunoprecipitates were prepared with anti-Flag antibody and separated by SDS-PAGE. Following transfer, the membrane was exposed to a film (top) and detected with anti-Flag antibody (bottom).

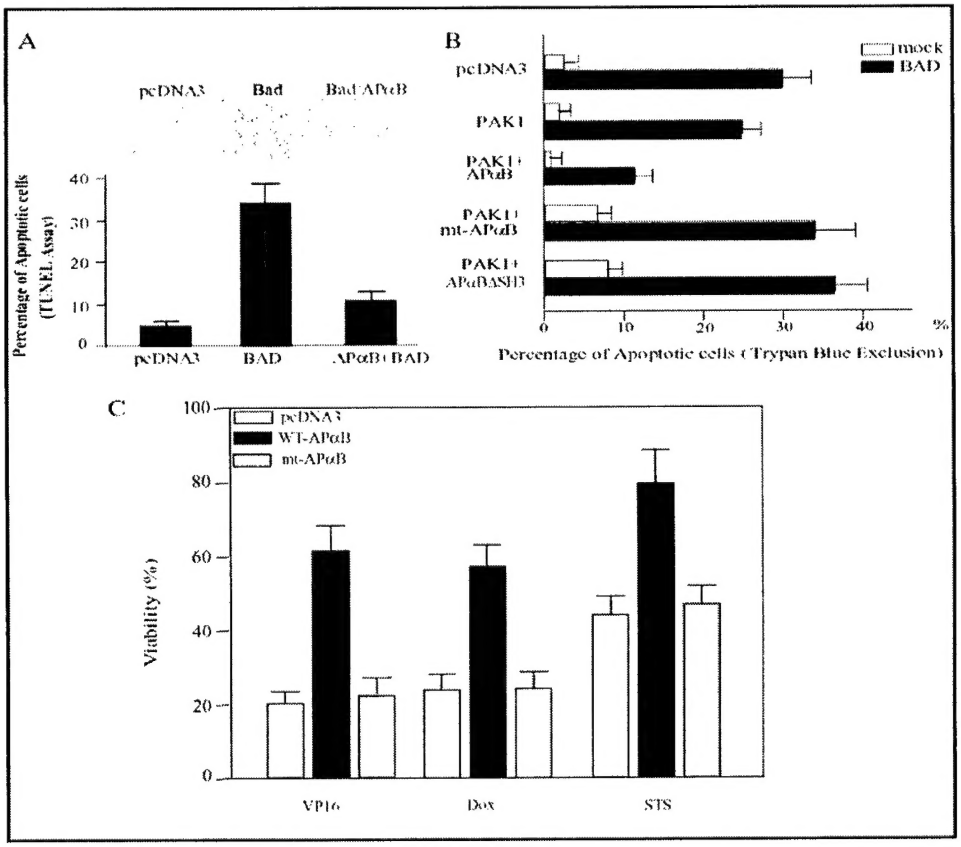

Fig. 11. APαB protects cell from apoptosis induced by Bad and DNA damage. (**A** and **B**) Tunel assay. HEK293 cells were transiently transfected with indicated expression constructs. Apoptotic cells were detected by Tunal assay and quantified after 48 h of the transfection. (**C**) Hela cells were stably transfected with wild type and nonphosphorylatable APαB. The cells transfected with pcDNA3 were used as control. After treatment with VP16, doxorubicin or staurosporine, apoptosis was evaluated with Tunal assay and quantified. Each experiment was repeated three times.

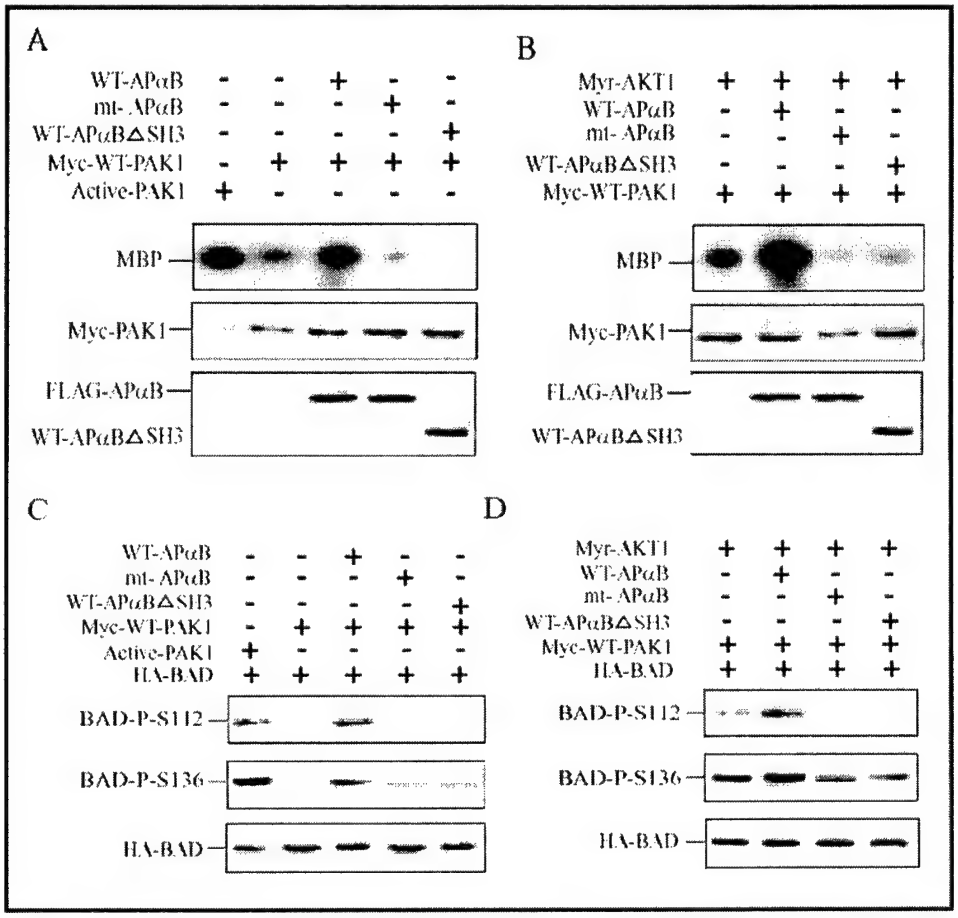

Fig. 12. APαB stimulates and mediates Akt-induced PAK1 activation. (**A** and **B**) *In vitro* PAK1 kinase assay. HEK 293 cells were transfected with indicated expression plasmids and immunoprecipitated with anti-Myc antibody. The Myc-PAK1 immunoprecipitates were subjected into in vitro kinase assay using MBP as substrate. Expression APαB alone is sufficient to activate PAK1 (A) and constitutively Akt and APαB exhibit synergetic effect on PAK1 activation (B). Panels 2 and 3 show the expression of transfected plasmids. (**C** and **D**) Western blot analysis of cell lysates prepared from HEK293 cells transfected with indicated plasmids. The blots were detected with anti-phospho-Bad-S112 (top), -S136 (medium) and anti-HA (bottom) antibodies.

Key Research Accomplishment

1 AKT2 contributes to human oncogenesis by inhibition of proapoptotic molecule MST1.
2 AKT2 phosphorylates MST1 leading to inhibition its cleavage, kinase activity and nuclear translocation.
3 JNK1 phosphorylation of MST1 enhances MST1 kinase activity and nuclear translocation which is inhibited by Akt.
4 AKT2 interaction protein, APBP/APαB is a substrate of Akt.
5 APBP/APαB mediates Akt activation of PAK1 to inactivate pro-apoptotic protein BAD,

Reportable Outcomes

1. Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, and Cheng JQ. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-13440, 2003.
2. Yuan Z, Feldman RI, Nicosia SV, and Cheng JQ. Akt Phosphorylates Mammalian Hippo Ortholog MST1 to Attenuate Apoptosis through Inhibition of its Cleavage and Kinase Activity. (Manuscript in preparation).
3. Yuan Z, Nicosia SV, and Cheng JQ. APBP/APαB, an Adaptor Protein, Interacts with Akt and p21-Activated Kinase-1 and Promotes Cell Survival (Manuscript in preparation).

Conclusion

1. MST1 is a physiological substrate of Akt.
2. Akt phosphorylates MST1 leading to inhibition of its cleavage, kinase activity and nuclear translocation and attenuation of MST1-induced apoptosis.
3. APBP/APαB mediates Akt survival signal by interaction of Akt and PAK1.

Reference

1. Harvey KF, Pfleger CM, Hariharan IK. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell,* 114:457-67, 2003.

2. Wu S, Huang J, Dong J, Pan D. Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell,* 114:445-56, 2003.

3. Hay BA, Guo M. Coupling cell growth, proliferation, and death. Hippo weighs in. *Dev Cell.* 5:361-363, 2003

4. Glantschnig H, Rodan GA, Reszka AA.. Mapping of MST1 kinase sites of phosphorylation. Activation and autophosphorylation. *J Biol Chem.* 277:42987-96, 2002.

Appendix:

1. Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, and Cheng JQ. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-13440, 2003.

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AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1

IMPLICATION OF AKT2 IN CHEMORESISTANCE*

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Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers. Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling downstream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects.

Although cisplatin and its analogues, the DNA cross-linking agents, are first-line chemotherapeutic agents for the treatment of human ovarian and breast cancers, chemoresistance remains a major hurdle to successful therapy (1, 2). Several molecules have been implicated in cisplatin resistance, including decreased cellular detoxication (3, 4), increased DNA repair (5), and mutations of *p53* tumor suppressor gene (6, 7). However, the mechanisms involved in cisplatin resistance are still poorly understood. A growing body of evidence indicates that defects in the intra- and extracellular survival/apoptotic pathways are an important cause of resistance to cytotoxic agents.

Phosphatidylinositol 3-kinase (PI3K)[1]/Akt is a major cell survival pathway that has been extensively studied recently (8). PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit and converts the plasma membrane lipid phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Pleckstrin homology domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Akt (also known as PKB) represents a subfamily of serine/threonine kinases. Three member of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated in a PI3K-dependent manner by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (9–12). Downstream targets of Akt contain the consensus phosphorylation sequence RXRXX(S/T)(F/L) (13). Several targets of Akt that have been identified have roles in the regulation of apoptosis, such as the proapoptotic proteins BAD and caspase-9 and transcription factor FKHRL1. Phosphorylation by Akt blocks BAD binding to Bcl-x_L, inhibits caspase-9 protease activity, and blocks FKHRL1 function, reducing Fas ligand transcription (14–16).

Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies including ovarian cancer. We have demonstrated previously amplification of the AKT2 in a number of human ovarian cancer cell lines and recently detected frequently elevated protein and kinase levels of AKT2 in about a half of primary ovarian carcinoma examined (17, 18). Moreover, ectopic expression of wild type of AKT2 but not Akt1 in NIH 3T3 cells resulted in malignant transformation (19). Inhibition of PI3K/AKT2 by farnesyltransferase inhibitor-277 induced apoptosis in ovarian cancer cells that overexpress AKT2 (20). We have also shown that TNFα and extracellular stresses, including UV irradiation, heat shock, and hyperosmolarity, induce AKT2 kinase and that

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¶ Predoctoral Fellowship awardee from the United States Department of Defense.

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; TNFα, tumor necrosis factor α; GST, glutathione *S*-transferase; HEK, human embryonic kidney; MKK, mitogen-activated protein kinase kinase.

activated AKT2 inhibits JNK/p38 activity to protect cells from TNFα and cellular stress-induced apoptosis (21).

JNK and p38 are predominantly activated through environmental stresses, including osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors, stimulation of Fas, and inflammatory cytokines such as TNFα and interleukin-1. Stimulation of JNK/p38 activity has also been shown to be critical for cisplatin-induced apoptosis in some cancer cells (22, 23). Specific inhibition of JNK or p38, through small molecule inhibitors, dominant negative JNK/p38 mutants, or knock-out of JNK expression, suppresses various types of stress-induced apoptosis (24). Although it has been shown that JNK phosphorylates and inhibits antiapoptotic protein Bcl-2 (25), the mechanism of JNK/p38 induction of apoptosis is still not well understood.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase family that activates both the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (26–28). ASK1 is a general mediator of cell death in responds to a variety of stimuli, including oxidative stress (29, 30) and chemotherapeutic drugs such as cisplatin and paclitaxel (22, 23). Ectopic expression of ASK1 induced apoptosis in various cell types (26, 28). Furthermore, disruption of the *ASK1* gene in mice causes a remarkable reduction in sensitivity to stress-induced cell death, such as that promoted by TNFα or oxidative stress (33). These data indicate that ASK1 plays a key proapoptotic function through promoting the sustained activation of JNK/p38 mitogen-activated protein kinases.

In the present study, we show that AKT2 activity promotes resistance to cisplatin-induced apoptosis in A2780S ovarian cancer cells through the inhibition of the ASK1/JNK/p38 pathway. In A2780S cells, we show that AKT2 complexes with and phosphorylates ASK1 at Ser-83 within a consensus Akt phosphorylation site on this molecule. This results in inhibition of ASK1 activity and the blocking of JNK and p38 activation. We also show that these latter activities are required for cisplatin-induced apoptosis in A2780S cells. Furthermore, in response to cisplatin, we observe that ASK1 and JNK/p38 promote Bax conformational change. Collectively, these studies indicate that AKT2 may be an important mediator of chemoresistance through its regulatory effects on the ASK1/JNK/p38/Bax pathway.

EXPERIMENTAL PROCEDURES

Reagents—Cisplatin, LY294002, and anti-Bax (6A7) were obtained from Sigma. DMEM and fetal bovine serum were purchased from Invitrogen. Anti-phospho-Akt (Ser-473), anti-cleaved PARP, anti-phospho-JNK (p54/44), anti-phospho-extracellular signal-regulated kinase 1/2(44/42), anti-phospho-p38, anti-phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2, and anti-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). GST-c-Jun and GST-ATF6 were also purchased from Cell Signaling. Anti-AKT2, anti-Bax, and anti-ASK1 were obtained from Santa Cruz Biotechnology. JNK inhibitor II and p38 inhibitor SB203580 were from Calbiochem.

Cell Culture and Cisplatin Treatment—The human epithelial cancer cell lines, A2780S and A2780CP, kindly provided by Benjamin K. Tsang at The Ottawa Hospital, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells per dish. After 24 h, cells were treated with cisplatin (20 μM) for the appropriate time as noted in the figure legends.

Expression Constructs—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2 and constitutively active HA-Myr-AKT2 have been described previously (31). The pcDNA$_3$-HA-ASK1 construct was kindly provided by Hidenori Ihijo at Tokyo Medical and Dental University. HA-ASK1-S83A and ASK1-S83D, as well as dominant negative AKT2 with triple mutations (T309A, E299K, and S474A), were created using the QuikChange site-directed mutagenesis kit (Stratagene). JNK and p38 plasmids were obtained from Roger Davis at the University of Massachusetts.

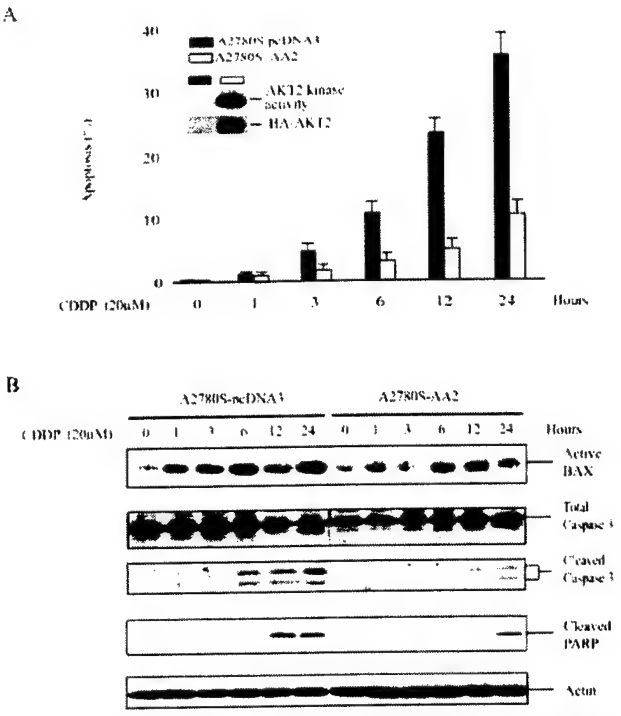

FIG. 1. **Activation of AKT2 renders cells resistant to cisplatin and inhibits cisplatin-induced Bax conformational change and caspase-3 cleavage.** *A*, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (*A2780S-AA2*) or pcDNA3 (*A2780S-pcDNA3*). Expression and kinase activity of transfected AKT2 were examined by Western blot and *in vitro* kinase assays (*inset*). The cells were treated with cisplatin (*CDDP*; 20 μM) for indicated time and analyzed by Tunel assay. Apoptotic cells were quantified in triple experiments. *B*, Western blot analysis. The cells were treated with cisplatin and lysed. A portion of lysate was immunoprecipitated with anti-active Bax (6A7) and detected with anti-total Bax antibody (*top panel*). The rest of the lysates were immunoblotted and probed with anti-caspase-3 (*second and third panels*), anti-PARP (*fourth panel*), and anti-actin (*bottom panel*) antibodies.

Immunoprecipitation and Immunoblotting—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerolphosphate. Lysates were centrifuged at $12,000 \times g$ for 15 min at 4 °C prior to immunoprecipitation or Western blot. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated with appropriate antibodies in the presence of 25 μl of protein A-protein G (2:1)-agarose beads for at least 2 h at 4 °C. The beads were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10; twice with phosphate-buffered saline; and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerolphosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the appropriate antibodies as noted in the figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting analysis system (Amersham Biosciences).

In Vitro Kinase Assay—Protein kinase assays were performed as described previously (21). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. 2 μg of myelin basic protein was used as the exogenous substrate. After incubation at room temperature for 30 min the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each

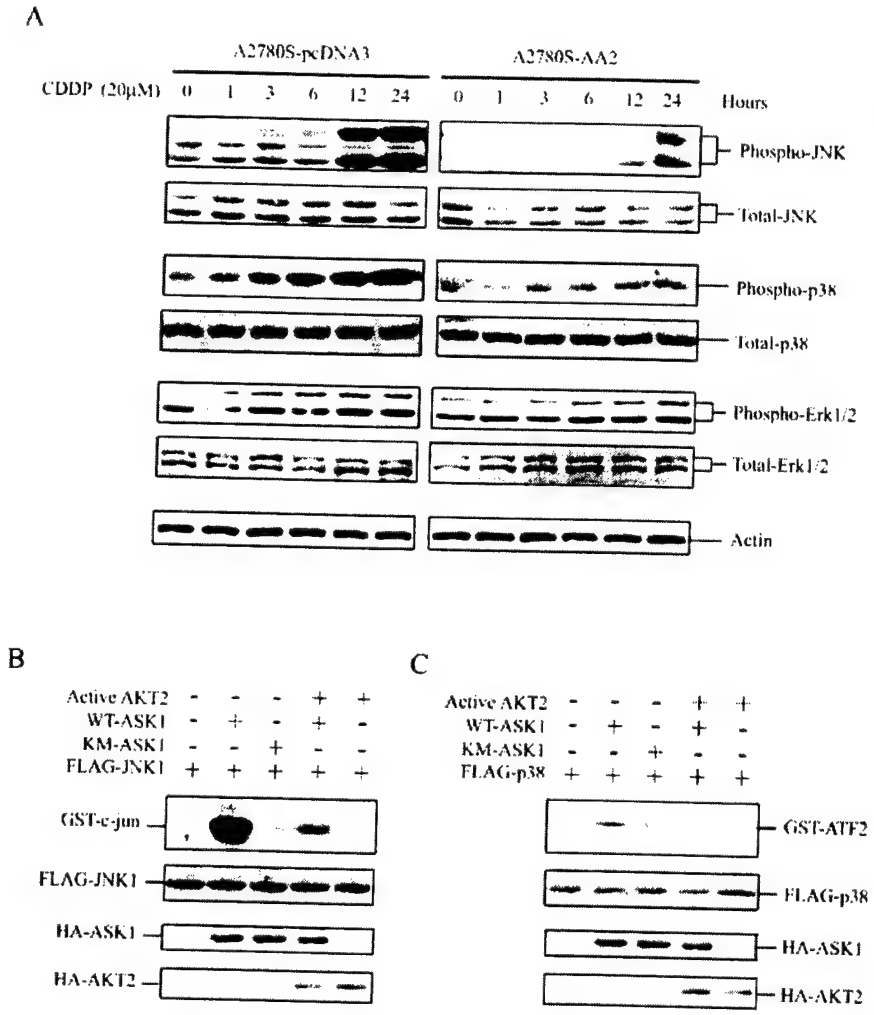

FIG. 2. **AKT2 inhibits JNK and p38 activation induced by cisplatin and ASK1.** *A*, immunoblotting analysis. Following treatment with cisplatin at indicated times, the cells were lysed and immunoblotted. The blots were detected with indicated antibodies. *B* and *C*, *in vitro* kinase assay. HEK293 cells were transfected with the indicated expression plasmids. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. The FLAG-JNK and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase using GST-c-Jun (*B*) and GST-ATF2 (*C*) as substrate, respectively (*top panel*). Expression of the transfected plasmids was shown in the *second*, *third*, and *fourth panels*.

experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

In Vivo [^{32}P]P$_i$ Labeling—HEK293 cells were co-transfected with active AKT2 and HA-tagged ASK1 or pcDNA3 and labeled with [^{32}P]P$_i$ (0.5 mCi/ml) in phosphate- and serum-free DMEM medium for 4 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to membranes. Phosphorylated ASK1 band was visualized by autoradiography. The expression of transfected ASK1 was detected with anti-HA antibody.

Luciferase Reporter Assay—Cells were seeded in 6-well plate and transfected with c-Jun or ATF6 reporter plasmid (pGl-GAL4), pSV2-β-gal, and different forms (wild type, constitutively active, or dominant negative) of HA-AKT2 together with or without different forms of ASK1 or vector alone. After 36 h of the transfection, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

Tunel Assay—Cells were seeded into 60-mm dishes and grown in DMEM supplemented with 10% fetal bovine serum for 24 h and treated with 20 μM cisplatin for different times. Apoptosis was determined by Tunel assay using an *in situ* cell death detection kit (Roche Applied Science). These experiments were performed in triplicate.

RESULTS

Activation of AKT2 Renders Cisplatin-sensitive Cells Resistant to Cisplatin and Inhibits Cisplatin-induced Bax Conformational Change—We have shown previously (18, 34) frequent activation of AKT2 kinase in human ovarian and breast cancers. To examine whether activation of AKT2 contributes to chemoresistance in cancer cells, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (A2780S-AA2) or pcDNA3 vector alone. Expression and kinase activity of transfected constitutively active AKT2 were confirmed by Western blot and *in vitro* kinase analysis (Fig. 1*A*, *inset*). Following treatment with cisplatin (20 μM) for 0, 1, 3, 6, 12, and 24 h, programmed cell death in A2780S-pcDNA3 and A2780S-AA2 (active AKT2) cells were examined by Tunel assay. The number of apoptotic cells was quantified by counting three different microscopic fields. Three h after treatment, A2780S-pcDNA3 cells begun to undergo apoptosis. By 24 h of treatment, 35% of the cells were apoptotic, which is a similar response reported in the literature for parental A2780S cells (35). However, we observed a distinctly lower percentage of apoptotic cells at the time points 3, 6, 12, and 24 h in A2780S-AA2 cells (Fig. 1*A*), indicating that activation of AKT2 renders cisplatin-sensitive A2780S cells resistant to cisplatin.

It has been shown that Bax is required for cisplatin-induced apoptosis, *i.e.* cisplatin activates Bax by inducing its N-terminal conformation change and then targeting it to mitochondria resulting in cytochrome *c* release and activation of apoptotic pathway (36, 37). Thus, we next examined the effects of AKT2 activation on induction of Bax conformational changes by cisplatin. After treatment with cisplatin, A2780S-pcDNA3 and A2780S-AA2 cells were lysed and immunoprecipitated with anti-active Bax (6A7) antibody. The immunoprecipitates were subjected to Western blot analysis with total anti-Bax anti-

body. As shown in Fig. 1*B*, cisplatin promotes alteration of Bax conformation after 3 h of treatment in A2780S-pcDNA3 cells but not in A2780S-AA2 cells. Accordingly, cleavage of caspase 3 and its substrate, PARP, was also inhibited by expression of constitutively active AKT2 as compared with pcDNA3-transfected A2780S cells (Fig. 1*B*).

AKT2 Inhibits Cisplatin- and ASK1-induced JNK and p38 Activation—It has been documented that stress kinases, JNK and p38, are activated by cisplatin, and their activations are required for cisplatin-induced programmed cell death (22, 23, 38). To examine whether the effect of cisplatin on JNK and p38 is abrogated by the activation of AKT2, A2780S-pcDNA3 and A2780S-AA2 cells were treated with cisplatin at different times. As expected, JNK and p38 were activated by cisplatin in A2780S-pcDNA3 cells, and the activation of p38 took place before that of JNK. However, the activation of JNK and p38 was reduced dramatically in A2780S cells transfected with a constitutively active AKT2. No significant difference in the phosphorylation levels of extracellular signal-regulated kinase was observed between these two cell lines (Fig. 2*A*).

To explore the mechanism of AKT2 inhibition of the JNK and p38, we probed for direct interaction of these proteins by coimmunoprecipitation. We were not, however, able to demonstrate any interaction between AKT2 and JNK or p38 (data not shown). As ASK1 is known to activate JNK/p38 and be induced by cisplatin (32), and its overexpression is sufficient to induce apoptosis (26, 28), we next examined whether AKT2 restrains JNK and p38 activity through inhibition of ASK1. HEK293 cells were transfected with FLAG-JNK1 or FLAG-p38 and wild type or kinase-dead ASK1 (KM-ASK1), with or without constitutively active AKT2. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. FLAG-JNK1 and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase assays using GST-c-Jun and GST-ATF2 as substrates, respectively. Repeated experiments revealed that kinase activities of JNK1 and p38 were significantly induced by expression of wild type but not kinase-dead ASK1 and that the activation of JNK and p38 was attenuated by ectopic expression of constitutively active AKT2 (Fig. 2, *B* and *C*). These data indicate that AKT2 may negatively regulate ASK1, causing inhibition of cisplatin-induced JNK/p38 activation and apoptosis.

AKT2 Interacts with, Phosphorylates, and Inhibits ASK1—To examine whether ASK1 is a direct target of AKT2, co-immunoprecipitation was carried out with anti-AKT2 antibody and detected with anti-ASK1 antibody, and vice versa. As shown in Fig. 3, *A* and *B*, interaction between ASK1 and AKT2 was readily detected, and this interaction was enhanced by cisplatin treatment. Sequence analysis revealed that an AKT2 phosphorylation consensus site resides in ASK1 at residue Ser-83, which is conserved between human and mouse. To determine whether AKT2 phosphorylates ASK1, *in vitro* AKT2 kinase assays were performed using immunoprecipitated HA-ASK1 (wild type ASK1 or ASK1S83A) as substrates (Fig. 3*C*). In addition, *in vivo* [^{32}P] labeling and immunoblotting analyses with anti-phospho-Ser/Thr Akt substrate antibody were carried out in HEK293 cells transfected with ASK1 and constitutively active or wild type AKT2 (Fig. 3*D*). Both *in vitro* kinase and *in vivo* labeling experiments, as well as Western blot analysis, showed that wild type and constitutively active AKT2 phosphorylate ASK1 at Ser-83 with the lower phosphorylation level by wild type AKT2 (Fig. 3, *C* and *D*).

We next determined whether cisplatin-induced ASK1 activation is inhibited by AKT2 and, if it is, whether this inhibition depends upon AKT2 phosphorylation of ASK1 at Ser-83. Mutagenesis was used to create a form of ASK1 not phosphorylatable by AKT2, ASK1-S83A, prepared by converting Ser-83 of ASK1 to alanine. We also prepared ASK1-S83D, derived from mutating Ser-83 of ASK1 to aspartic acid, which mimics ASK1 phosphorylated by AKT2. A2780S cells were transfected with ASK1-S83A or ASK1-S83D, with or without constitutively active AKT2. Following cisplatin treatment, ASK1s were immunoprecipitated, and *in vitro* ASK1 kinase assays were performed using myelin basic protein as substrate. As shown in Fig. 3*E*, cisplatin significantly induced the kinase activity of both wild type ASK1 and nonphosphorylatable ASK1-S83A but not AKT2 phosphomimic ASK1-S83D. Expression of constitutively active AKT2 inhibited cisplatin-stimulated kinase activity of wild type ASK1 but not that of nonphosphorylatable ASK1-S83A. These results indicate that ASK1 kinase activity is negatively regulated by AKT2 through phosphorylation of ASK1 at Ser-83.

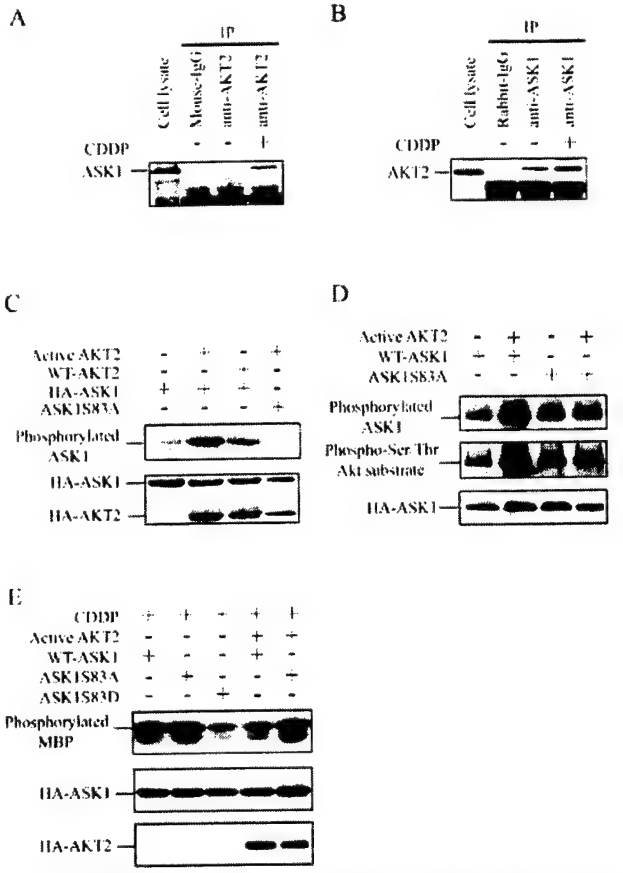

FIG. 3. **AKT2 interacts with and phosphorylates ASK1 and inhibits ASK1 kinase activity.** *A* and *B*, Western blot analyses of the immunoprecipitates prepared from A2780S cells treated with or without cisplatin. Immunoprecipitation was performed with anti-AKT2 and detected with anti-ASK1 antibody (*A*) and vice versa (*B*). *C*, *in vitro* kinase analysis of AKT2 immunoprecipitates derived from HEK293 cells that were transfected with indicated plasmids. Immunopurified HA-ASK1 or HA-ASK1-S83A was used as substrate (*top panel*). *The bottom panel* shows expression of transfected plasmids. *D*, *in vivo* [^{32}P]P_i labeling. HEK293 cells were transfected with indicated expression constructs, labeled with [^{32}P]P_i (0.5 mCi/ml), and immunoprecipitated with anti-HA antibody. The HA-ASK1 immunoprecipitates were separated in SDS-PAGE, blotted, and exposed to x-ray film (*top panel*). The membrane was then detected with anti-Akt substrate antibody (*middle panel*) and anti-HA antibody (*bottom panel*). *E*, *in vitro* ASK1 kinase analysis of the immunoprecipitates prepared from A2780S cells transfected with indicated plasmids and treated with cisplatin (20 μM) for 6 h. Myelin basic protein was used as substrate (*top panel*). Expression of transfected different forms of ASK1 and AKT2 was shown in the *second and third panels*.

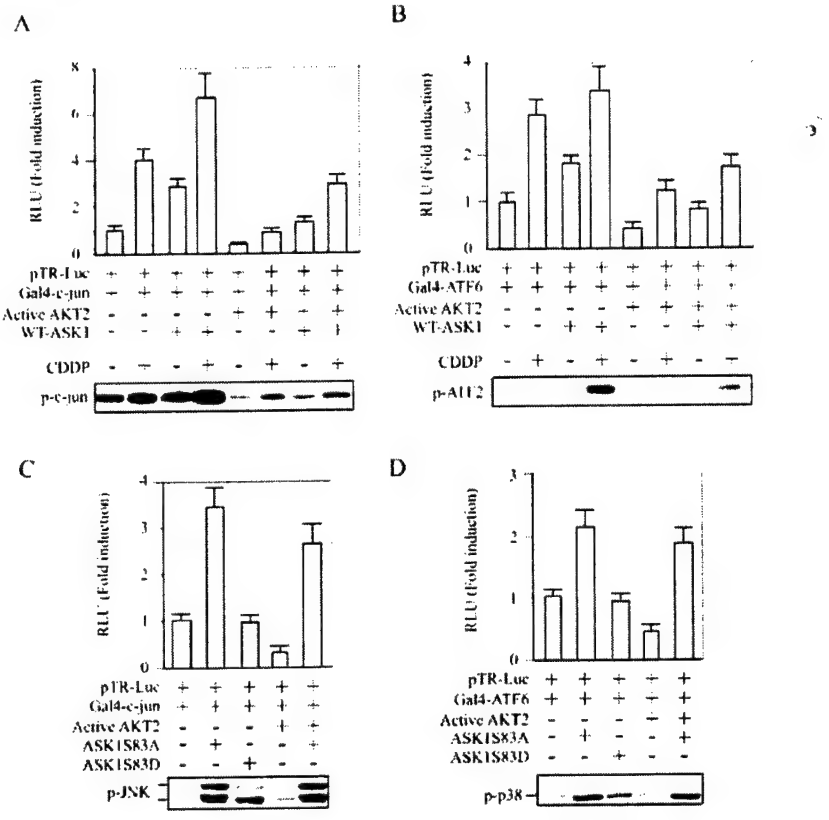

FIG. 4. **Activation of AKT2 inhibits ASK1- and/or cisplatin-induced JNK and p38 activation.** *A–D*, luciferase reporter assays. A2780S cells were transfected with indicated expression constructs and treated with or without cisplatin. Luciferase and β-galactosidase assays were performed, and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. Each experiment was repeated three times. The *bottom panels* of *A* and *B* show the results obtained from *in vitro* JNK and p38 kinase assays using GST-c-Jun and ATF2 as substrates, respectively. The effects of AKT2 and its phosphorylation of ASK1 at Ser-83 on JNK and p38 activation were shown in the *bottom panels* of *C* and *D*.

AKT2 Inhibition of Cisplatin-stimulated JNK and p38 Is Mediated by Phosphorylation of ASK1 at Residue Ser-83—We next determined whether phosphorylation of ASK1 on Ser-83 by AKT2 is required for AKT2 inhibition of p38 and JNK, which are downstream from ASK1. Luciferase reporter assays were performed using Gal4-c-Jun/pTR-Luc (for JNK) and Gal4-ATF6/pTR-Luc (for p38) reporter systems. A2780S cells were transfected with ASK1, ASK1-S83A, ASK1-S83D, and/or Myr-AKT2, as well as pTR-Luc, Gal4-c-Jun, or Gal4-ATF6, and treated with or without cisplatin. Three independent experiments revealed that cisplatin induces Gal4-c-Jun or Gal4-ATF-regulated reporter activities. Further, *in vitro* JNK and p38 kinase analysis revealed that the phosphorylation of c-Jun and ATF2 was also stimulated by cisplatin treatment. These effects were enhanced by ectopic expression of wild type ASK1; however, they were inhibited by expression of constitutively active AKT2 (Fig. 4, *A* and *B*). Expression of nonphosphorylatable ASK1-S83A was also sufficient to induce the reporter activities and to attenuate the inhibitory action of constitutively active AKT2. In contrast, phosphomimic ASK1-S83D failed to stimulate the reporter activities (Fig. 4, *C* and *D*). Moreover, the effects of ASK1-S83A and ASK1-S83D on cisplatin-induced JNK and p38 activation were similar to their action on Gal4-c-Jun and Gal4-ATF6 reporters (Fig. 5*A*). Therefore, we conclude that AKT2 inhibits cisplatin-induced JNK and p38 via a phosphorylation of ASK1-dependent manner.

Cisplatin-induced Bax Conformational Change Is Regulated by AKT2 Phosphorylation of ASK1—Previous studies have shown that JNK is required for UV- and cisplatin-induced Bax conformational change (39). Our data demonstrate that ectopic expression of constitutively active AKT2 overrides cisplatin-induced ASK1/JNK/p38 activation and prevents formation of the active Bax conformation (see Figs. 1 and 2). To more directly probe the effect of AKT2 phosphorylation of ASK1 on Bax activation, we transfected A2780S cells with nonphosphorylatable and phosphomimic ASK1 and treated the cells with or without cisplatin. As revealed by immunoprecipitation and Western blot analyses, ectopic expression of nonphosphorylatable ASK1-S83A enhance cisplatin-dependent Bax conformational change, whereas ASK1-S83D, mimicking ASK1 phosphorylated by AKT2, inhibited cisplatin-induced Bax

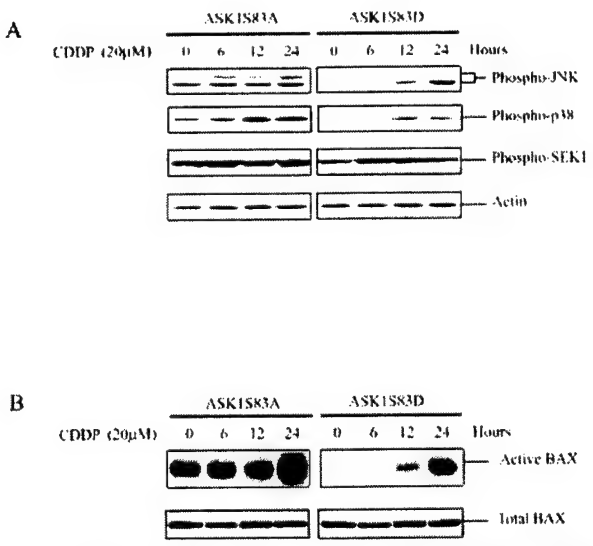

FIG. 5. **AKT2 phosphorylation of ASK1 at Ser-83 plays a critical role in cisplatin-induced JNK/p38 activation and Bax conformational change.** *A*, immunoblotting analysis of A2780S cells transfected with nonphosphorylatable and phosphomimic ASK1 prior to treatment with cisplatin. The blots were probed with the indicated antibodies. *B*, Western blot analysis. A2780S cells were transfected with indicated expression plasmids, treated with cisplatin, immunoprecipitated with anti-active Bax antibody, and detected with anti-total Bax antibody (*top panel*). Expression of Bax was shown in the *bottom panel*.

activation (Fig. 5*B versus* Fig. 1*B*). These data suggest that AKT2 inhibition of cisplatin-stimulated Bax conformational change is mediated at least to some extent by AKT2 phosphorylation of ASK1 at residue Ser-83.

Because JNK and p38 are downstream targets of ASK1, we next examined their roles in ASK1-stimulated Bax activation by using selective small molecule inhibitors of JNK and p38, JNK inhibitor II and SB 203580. As illustrated in Fig 6*A*, expression of ASK1 was sufficient to induce a Bax conformational change, and this effect was enhanced by cisplatin treatment. However, the conformational change of Bax induced by ASK1 and/or cisplatin was significantly diminished following treatment of cells with JNK inhibitor II (10 μM) and p38 inhibitor, SB 203580 (10 μM), suggesting that JNK and/or p38 mediate cisplatin-induced Bax activation. To probe the individual contributions of JNK and p38 in cisplatin-stimulated Bax activation, we further examined the effects of small molecule inhibitors of p38 and JNK and the expression of wild type and dominant negative forms of these kinases. A2780S cells were transfected with wild type or dominant negative JNK or p38, together with ASK1, and treated with or without cisplatin and/or inhibitor of JNK or p38. As shown in Fig. 6, *B* and *C*, expression of wild type JNK or p38 enhanced ASK1- and cisplatin-induced Bax activation, as expected. Furthermore, dominant negative JNK or a small molecule JNK inhibitor significantly decreased the Bax activation induced by cisplatin treatment or ectopic expression of ASK1 (Fig. 6*B*). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6*C*). These results indicate that cisplatin- and/or ASK1-induced Bax activation is mediated primarily by JNK.

Inhibition of PI3K/AKT2 Pathway Sensitizes Cells to Cisplatin-induced Apoptosis—Because activated AKT2 reduces the cisplatin sensitivity of A2780S cells, we next examined the ability of inhibition of the PI3K/AKT2 pathway to sensitize cells to cisplatin-induced apoptosis. Cisplatin-resistant A2780CP and A2780S cells were transfected with dominant negative AKT2 or treated with PI3K inhibitor, LY294002, together with cisplatin. Tunel assay analyses revealed that either LY294002 or ectopic expression of dominant negative AKT2 enhanced cisplatin-induced apoptosis as compared with cells treated with cisplatin alone (Fig. 7, *A* and *C*). Accordingly, cleavage of caspase-3 and PARP was increased by treatment of cells with a combination of cisplatin with LY294002 or dominant negative-AKT2 (Fig. 7, *B* and *D*). To examine the role played by AKT2 phosphorylation of ASK1 in cisplatin-induced apoptosis, we transfected A2780S cells with ASK1-S83A, which is not phosphorylated by AKT2, ASK1-S83D, which mimics AKT2 phosphorylation, and then induced apoptosis with cisplatin. Notably, ectopic expression of ASK1-S83A significantly augmented cisplatin-induced apoptosis. In contrast, expression of ASK1-S83D conferred resistance to cisplatin (Fig. 7*E*). These data further indicate that PI3K/AKT2 promotes cell survival through phosphorylation and inhibition of ASK1 signaling.

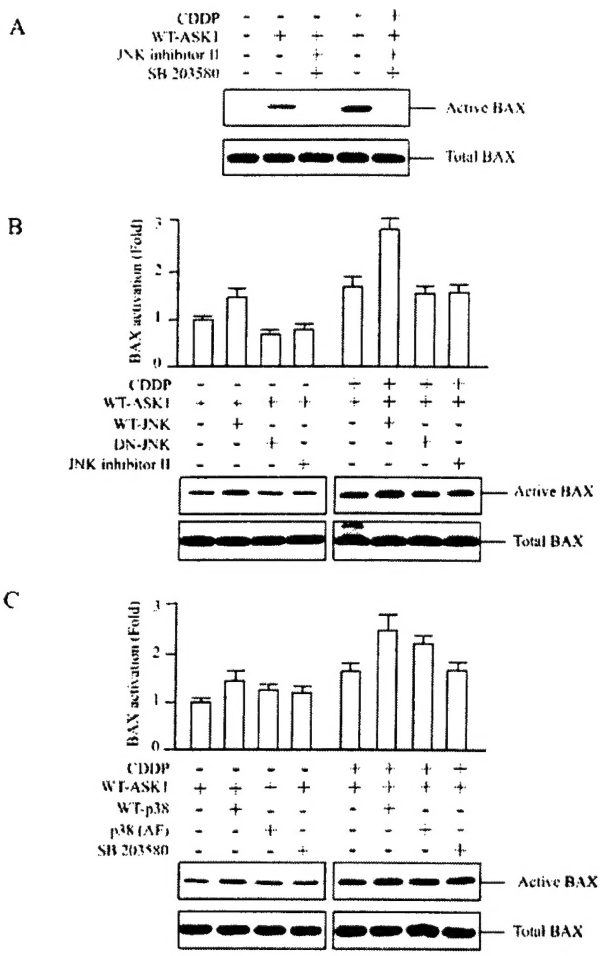

FIG. 6. **JNK and p38 mediate cisplatin- and ASK1-induced Bax conformational change.** *A*, Western blot analysis. A2780S cells were transfected with ASK1 and treated with JNK inhibitor II (10 μM) and SB 203580 (10 μM) for 1 h prior to addition of cisplatin. Following 16 h of the further treatment, Bax conformational change was examined as described above. *B* and *C*, immunoblotting analyses. A2780S cells were transfected with indicated plasmids and treated with indicated reagents. Bax conformational change was evaluated as described above. Both JNK inhibitor and dominant negative JNK exhibited more significant inhibitory effects on Bax activation than did p38 inhibitor and dominant negative p38 (AF). All the experiment was repeated three times.

DISCUSSION

We have demonstrated previously (18, 34) that AKT2 kinase is frequently elevated in human ovarian and breast cancers and that AKT2, like Akt1, exerts its anti-apoptotic function through phosphorylation of Bad (20). However, the biological role of AKT2 activation in human cancer and the mechanism of AKT2-induced cell survival in a chemotherapeutic setting have not been well documented. In this study, we show that activation of AKT2 significantly increases the resistance of ovarian cancer cells to cisplatin. AKT2 protects cells from cisplatin-induced apoptosis by inhibiting cisplatin-induced JNK/p38 activation and Bax conformational change. AKT2 mediates these effects through its interaction and phosphorylation of ASK1.

Cisplatin-induced JNK and p38 activations are required for its anti-tumor activity (22, 23). This activation has been shown to correlate with induction of apoptosis by cisplatin (22, 23). Moreover, studies using dominant negative mutants of JNK and p38 and specific pharmacological inhibitors have shown that activation of JNK and/or p38 is necessary for stress or chemotherapeutic drug-induced apoptosis (38, 40). Also, studies on fibroblasts with targeted disruptions of all the functional *Jnk* genes established an essential role for JNK in UV- and other stress-induced apoptosis (41). ASK1, an upstream regulator of JNK/p38, has also been shown to be induced by cisplatin (32). Furthermore, oxidative stress-induced ASK1 kinase activity is inhibited by Akt1 (42). Consistent with this, we demonstrate that activation of AKT2 inhibits cisplatin-induced JNK and p38 through direct interaction with and phosphorylation of ASK1 at serine 83. We also demonstrate that phosphorylation of ASK1 by AKT2 renders cells resistant to cisplatin.

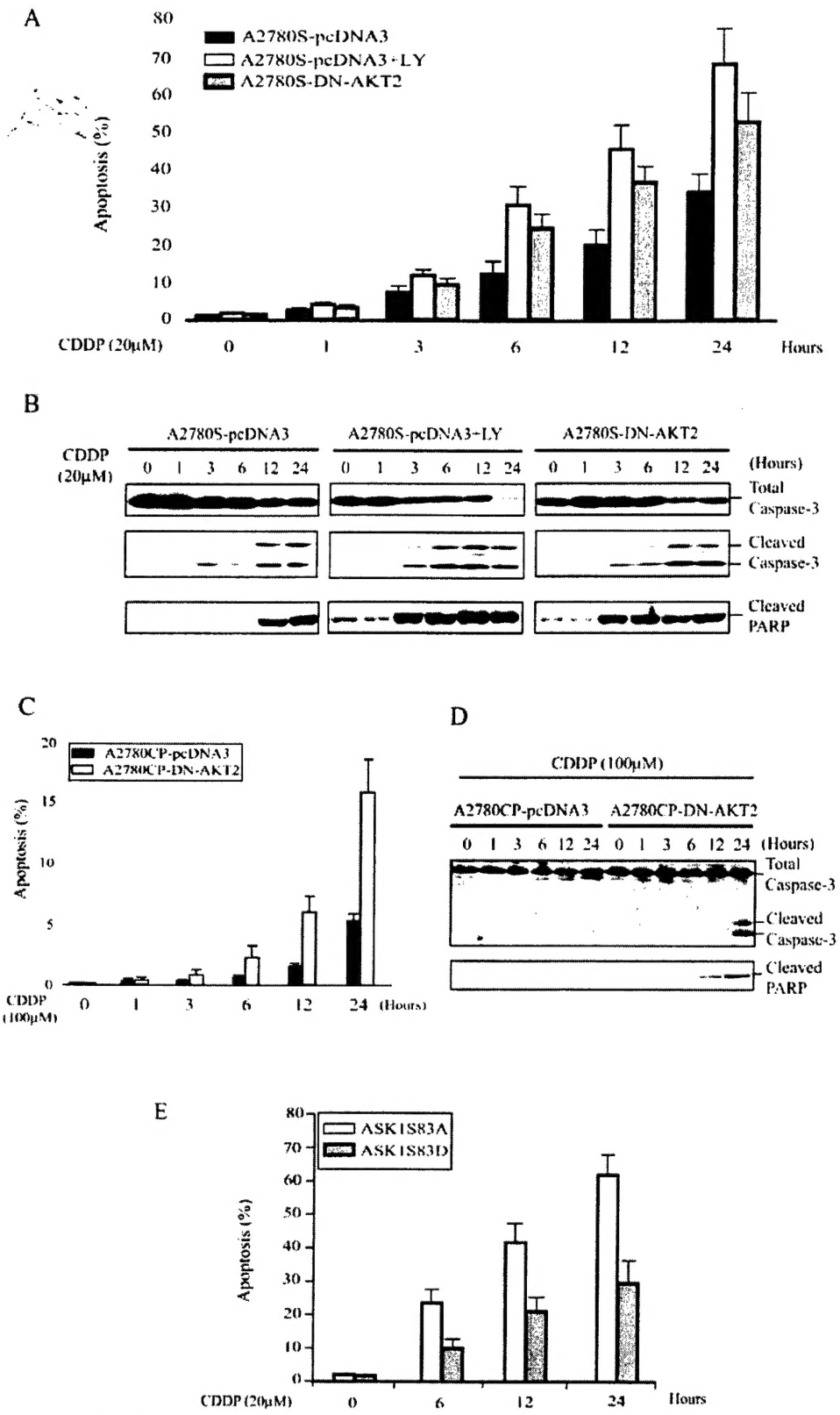

FIG. 7. **Inhibitions of PI3K/AKT2 and ASK1 phosphorylation sensitize cells to cisplatin-induced apoptosis.** *A*, Tunel assay. A2780S cells were transfected with dominant negative AKT2 or pcDNA3 vector and treated with cisplatin or cisplatin/LY294002. Apoptosis was examined and quantified after treatment for the indicated times. *B*, immunoblotting analysis of cell lysates prepared from cells treated as *A*. The blots were probed with indicated antibodies. *C* and *D*, cisplatin-resistant A2780CP cells transfected, treated, and analyzed as described in *A* and *B* except LY294002 treatment. *E*, Tunel assay. A2780S cells were transfected with indicated plasmids and treated with cisplatin. All the experiments were performed in triplicate.

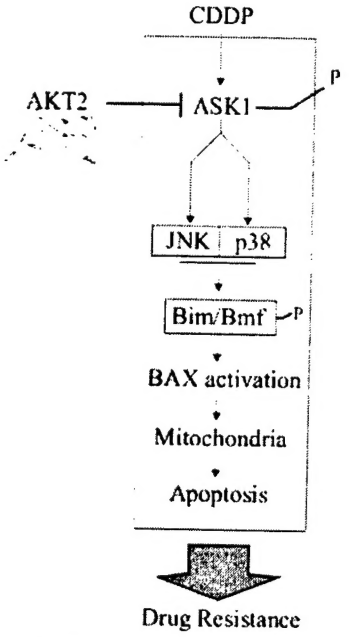

FIG. 8. **Schematic illustration of AKT2 regulation of ASK1/JNK/p38 and Bax.**

Besides the direct inhibition of ASK1, AKT2 could regulate JNK and p38 through other mechanisms. For example, NFκB-induced X chromosome-linked inhibitor of apoptosis and GADD45β down-regulate TNFα-induced JNK signaling (43, 44). We have demonstrated previously (21) that AKT2 inhibits UV- and TNFα-induced JNK and p38 by activation of the NFκB pathway (21). Therefore, we examined the possibility of AKT2 up-regulation of X chromosome-linked inhibitor of apoptosis and GADD45β. Western and Northern blot analyses, however, revealed no difference in X chromosome-linked inhibitor of apoptosis and GADD45β expression in A2780S cells transfected with constitutively active AKT2 or the control plasmid, pcDNA3 (data not shown). The possible reason is that cisplatin, unlike UV and TNFα, is incapable of inducing the NFκB pathway in A278S cells. In fact, our reporter assay revealed that cisplatin inhibits rather than activates NFκB activity in A2780S cells (data not shown).

In the present study, we observed that the ability of AKT2 to inhibit cisplatin-induced JNK/p38 was attenuated by nonphosphorylatable ASK1-S83A. Expression of phosphomimic ASK1-S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). In addition, ASK1-S83D exhibited effects similar to that of constitutively active AKT2, *i.e.* rendered cells resistant to cisplatin, whereas ASK1-S83A sensitized cells to cisplatin-induced apoptosis (Fig. 7*E*). Thus, we conclude that AKT2 inhibition of cisplatin-stimulated JNK/p38 activation leading to cisplatin resistance is mediated by AKT2 phosphorylation/inhibition of ASK1.

It has been demonstrated that cisplatin-induced Bax conformational change is also important for cisplatin-stimulated apoptosis (45). Bax is a pro-apoptotic member of the Bcl2 family. Accumulated evidence shows that death signals, including cisplatin, induce a conformational change of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and cytochrome *c* release (46, 47). Recent studies from Bax and/or Bak knock-out cells have shown that BH3-only proteins, such as tBid, Bad, Puma, and Bim, are required for inducing the activation of Bax and Bak by their direct interaction (48). Moreover, Akt has been shown to effectively inhibit Bax conformational change and contribute to chemoresistance (49). However, the mechanism by which Akt blocks Bax activation is poorly documented. We demonstrate in this report that ASK1 mediates at least in part cisplatin-induced Bax conformational change. Ectopic expression of constitutively active AKT2 attenuates cisplatin-induced Bax activation by phosphorylation and inhibition of ASK1. Downstream targets of ASK1, JNK, and p38, especially JNK, mediate AKT2 inhibition of Bax conformational change. These results are consistent with the recent findings obtained from a *Jnk*-deficient cell model (39).

Accumulated evidence shows that AKT2 plays a more significant role in human oncogenesis than AKT1 and AKT3. Frequent alterations of AKT2, but not AKT1 and AKT3, were detected in human cancers (18). Further, ectopic expression of AKT2, but not AKT1 and AKT3, leads to increased invasion and metastasis of human breast and ovarian cancer cells (50) and to malignant transformation of mouse fibroblasts (19). We observed in this study that A2780S cells expressing constitutively active AKT2 became cisplatin-resistant whereas expression of dominant negative AKT2 or treatment with PI3K inhibitor sensitized both cisplatin-sensitive (A2780S) and -resistant (A2780CP) ovarian cancer cells to cisplatin-induced apoptosis. Moreover, cisplatin-induced programmed cell death was enhanced by the expression of AKT2 nonphosphorylatable ASK1-S83A, whereas it is inhibited by phosphomimic ASK1-S83D. These data, therefore, indicate that activation of AKT2 contributes to cisplatin resistance by regulation of the ASK1/JNK/p38/Bax pathway and that the PI3K/AKT2/ASK1 cascade could be a critical therapeutic target for human cancer (Fig. 8).

A recent report (51) demonstrates that JNK and p38 phosphorylate BH3-only proapoptotic proteins Bim and Bmf, which was thought to mediate UV-induced apoptosis through a Bax-dependent mitochondrial apoptotic pathway (Fig. 8). Further investigation is required to determine the molecular mechanism by which ASK1/JNK/p38 regulates Bax activation in ovarian cancer cells, *i.e.* whether ASK1 and/or cisplatin induce Bim and Bmf phosphorylation and whether the phosphorylation is inhibited by PI3K/AKT2 pathway.

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REFERENCES

1. Charlotte, M., and Stan, B. K. (2002) *Eur. J. Cancer* **38,** 1701–1707
2. Ozoles, R. F., and R. C. Young. (1991) *Semin. Oncol.* **11,** 251–263
3. Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Behrens, B. C., Tsuruo, T., Grotzinger, K. R., McKoy, W. M., Young, R. C., and Ozols, R. F. (1985) *Biochem. Pharmacol.* **34,** 2583–2586
4. Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C., and Anderson, M. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3070–3074
5. Parker, R. J., Eastman, A., Bostic-Burton, F., and Reed, E. (1991) *J. Clin. Invest.* **87,** 772–777
6. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T, Zhang, W. W., Owen-Schaub, L. B., and Roth, J. A. (1994) *Cancer Res.* **54,** 2287–2291
7. Vikhanskaya, F., Erba, E., D'Incalci, M., and Broggini, M. (1994) *Nucleic Acids Res.* **22,** 1012–1017
8. Cantley, L. C. (2002) *Science* **296,** 1655–1657
9. Franke, T. F., Yang, S. L., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81,** 727–736
10. Burgering, B. M. T., and Coffer, P. J. (1995) *Nature* **376,** 599–602
11. Shaw, M., Cohen, P., and Alessi, D. R. (1998) *Biochem. J.* **336,** 241–246
12. Liu, A.-X., Testa, J. R., Hamilton, T. C., Jove, R., Nicosia, S. V., and Cheng, J. Q. (1998) *Cancer Res.* **58,** 2973–2977
13. Alessi, D. R., and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8,** 55–62
14. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, C. R., and Nunez, G. (1997) *Science* **278,** 687–689
15. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282,** 1318–1321
16. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L.-S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857–868
17. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 9267–9271
18. Yuan, Z. Q., Sun, M., Feldman, R. I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V., and Cheng, J. Q. (2000) *Oncogene* **19,** 2324–2330

19. Cheng, J. Q., Altomare, D. A., Klein, M. A., Lee, W. C., Kruh, G. D., Lissy, N. A., and Testa, J. R. (1997) *Oncogene* **14,** 2793–2801
20. Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S. V., Hamilton, A. D., Sebti, S. M., and Cheng, J. Q. (2000) *Mol. Cell. Biol.* **20,** 139–148
21. Yuan, Z. Q., Feldman, R. I., Sun, M., Olashaw, N. E., Coppola, D., Sussman, G. E., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2002) *J. Biol. Chem.* **277,** 29973–29982
22. Benhar, M., Dalyot, I., Engelberg, D., and Levitzki, A. (2001) *Mol. Cell. Biol.* **21,** 6913–6926
23. Sanchez-Perez, I., Murguia, J. R., and Perona, R. (1998) *Oncogene* **16,** 533–540
24. Tibbles, L. A., and Woodgett, J. R. (1999) *Mol. Life Sci.* **55,** 1230–1254
25. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. (1999) *Mol. Cell. Biol.* **19,** 8469–8478
26. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) *Science* **275,** 90–94
27. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) *Mol. Cell* **2,** 389–395
28. Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998) *Science* **281,** 1860–1863
29. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17,** 2596–2606
30. Gotoh, Y., and Cooper, J. A. (1998) *J. Biol. Chem.* **273,** 17477–17482
31. Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., and Wimalasena, J. (1998) *J. Biol. Chem.* **273,** 4928–4936
32. Chen, Z., Seimiya, H., Naito, M., Mashima, T., Kizaki, A., Dan, S., Imaizumi, M., Ichijo, H., Miyazono, K., and Tsuruo, T. (1999) *Oncogene* **18,** 173–180
33. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) *EMBO Rep.* **2,** 222–228
34. Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V., and Cheng, J. Q. (2001) *Am. J. Pathol.* **159,** 431–437
35. Asselin, E., Mills, G. B., and Tsang, B. K. (2001) *Cancer Res.* **61,** 1862–1868
36. Sugimoto, C., Fujieda, S., Seki, M., Sunaga, H., Fan, G. K., Tsuzuki, H., Borner, C., Saito, H., and Matsukawa, S. (1999) *Int. J. Cancer.* **82,** 860–867
37. Makin, G. W., Corfem B. M., Griffiths, G. J., Thistlethwaite, A., Hickman, J. A., and Dive, C. (2001) *EMBO J.* **20,** 6306–6315
38. Sanchez-Perez, I., and Perona, R. (1999) *FEBS Lett.* **453,** 151–158
39. Lei, K., Nimnual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D., and Davis, R. J. (2002) *Mol. Cell. Biol.* **22,** 4929–4942
40. Wang, X., Martindale, J. L., Liu, Y., and Holbrook, N. J. (1998) *Biochem. J.* **333,** 291–300
41. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288,** 870–874
42. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) *Mol. Cell. Biol.* **21,** 893–901
43. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) *Nature* **414,** 308–313
44. Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001) *Nature* **414,** 313–317
45. Devarajan, P., Savoca, M., Castaneda, M. P., Park, M. S., Esteban-Cruciani, N., Kalinec, G., and Kalinec, F. (2002) *Hearing Res.* **174,** 45–54
46. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) *EMBO J.* **17,** 3878–3885
47. Bellosillo, B., Villamor, N., Lopez-Guillermo, A., Marce, S., Bosch, F., Campo, E., Montserrat, E., and Colomer, D. (2002) *Blood* **100,** 1810–1816
48. Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N. R. (2002) *Mol. Cell. Biol.* **22,** 3577–3589
49. Yamaguchi, H., and Wang, H. G. (2001) *Oncogene* **20,** 7779–7786
50. Arboleda, M. J., Lyons, J. F., Kabbinavar, F. F., Bray, M. R., Snow, B. E., Ayala, R., Danino, M., Karlan, B. Y., and Slamon, D. J. (2003) *Cancer Res.* **63,** 196–206
51. Lei, K., and Davis, R. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2432–2437